Kidney International, Vol. 6 (1974), p. 241–246

Evidence for renal glomerular receptors for angiotensin II

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Evidence for renal glomerular receptors for angiotensin II. Monoiodinated angiotensin II (2000 mCi/μmole) was found to bind specifically to isolated rat glomeruli. Equilibrium was reached after 12 min and specific binding represented more than 95% of total binding. Dissociation after addition of an excess of unlabelled molecules was rapid. The k_1 and k_{-1} association and dissociation constants determined from time-course studies were $0.254 \pm 0.078 \times 10^{10}$ M^{-1} min^{-1} and 0.102 ± 0.018 min^{-1}, respectively and the ratio k_{-1}/k_1 (K_D) was $4.51 \pm 0.55 \times 10^{-11}$ M. Angiotensin II, liberated from glomerular binding sites at low pH, retained the ability to bind to fresh membranes. Angiotensin II, angiotensin I, ileu^8-angiotensin II and sarc^1-ileu^8-angiotensin II were all equally effective as competitive inhibitors of ^{125}I-angiotensin binding. In a preparation of isolated rat glomeruli, mean glomerular diameter decreased as a function of ^{125}I-angiotensin II concentration according to a sigmoidal effect vs. log dose curve and the calculated K_D (6×10^{-11} M) was similar to that obtained from binding studies. Specific binding of angiotensin II at physiological plasma concentration to rat glomeruli and correlation of this binding with glomerular vasoreactivity suggest a physiological role for this hormone in regulation of glomerular filtration.

Récepteurs rénaux glomérulaires de l'angiotensine II. L'Angiotensine II monoiodée (2000 mCi/μmole) se lie spécifiquement aux glomérules de rat isolés. L'équilibre est atteint au bout de 12 mn et la fixation spécifique représente plus de 95% de la fixation totale. L'addition d'un excès d'angiotensine non marquée détermine une dissociation rapide de l'hormone fixée. Les constantes d'association (k_1) et de dissociation (k_{-1}) calculées à partir des expériences de fixation en fonction du temps sont égales à $0{,}254 \pm 0{,}078 \times 10^{10}$ M^{-1} mn^{-1} et $0{,}102 \pm 0{,}018$ mn^{-1}, respectivement. Le rapport k_{-1}/k_1 (K_D) est égal à $4{,}51 \pm 0{,}55 \times 10^{-11}$ M. L'angiotensine II, éluée à pH acide de ses sites glomérulaires de liaison, garde la capacité de se lier de nouveau à des membranes fraîches. L'angiotensine II, l'angiotensine I, la ileu^8-angiotensine II et la sarc^1-ileu^8-angiotensine II sont des inhibiteurs compétitifs de la ^{125}I-angiotensine II, tous de même efficacité. Le diamètre glomérulaire moyen mesuré dans une préparation de glomérules isolés décroît en fonction de la concentration de la ^{125}I-angiotensine II selon une courbe sigmoïde (effet contre le log de la dose) et le K_D calculé (6×10^{-11} M) est semblable à celui obtenu à partir des expériences de liaison. La fixation spécifique aux glomérules de rat de l'angiotensine II à des concentrations identiques à celles présentes physiologiquement dans le plasma, ainsi que la corrélation entre la liaison de l'hormone aux glomérules et la vasomotricité glomérulaire suggèrent un rôle physiologique de l'angiotensine II dans la filtration glomérulaire.

Received for publication February 5, 1974;
and in revised form June 5, 1974.

Angiotensin II modifies urinary excretion of sodium in the rat [1] and may have a role in regulation of glomerular filtration [2]. Further, shrinkage of glomerular tufts has been observed under scanning microscopy after angiotensin II administration in the rat [3]. Glomerular receptors for angiotensin II have been postulated by Osborne et al [4] who, using autoradiography, observed localization of tritiated angiotensin II in rat mesangial cells after i.v. injection. This study presents evidence for specific angiotensin II receptors in rat glomeruli and for angiotensin II-induced mediation of glomerular vasoreactivity.

Methods

Materials. α-L-asparaginyl^1-valyl^5-angiotensin II was labelled with ^{125}I to a specific activity of the order of 50 μCi/μg by the method of Hunter and Greenwood [5] with minor modifications. Labelled and unlabelled molecules were separated using polyacrylamide gel electrophoresis according to the method of Corvol et al [6]. The specific activity of the labelled hormone was calculated in a radioimmunoassay system with progressively increasing doses of either labelled or unlabelled hormones. Linear transformation was obtained by plotting logit B/B_0 against $\log_e X$, where B_0 = bound hormone for zero dose, B = bound hormone for any dose and X = dose of either labelled or unlabelled angiotensin II. Statistical analysis as used in biological assays [7] demonstrated the linearity and parallelism of both curves and enabled calculation of the ratio cpm over pg which corresponded to 2200 mCi/μmole. This value is very close to the theoretical specific activity of one atom of iodine per mole of angiotensin II. Angiotensin I was obtained from Schwarz-Mann (Orangeburg, NY, U.S.A.). Sarc^1-ileu^8-angiotensin II and 1–34 bovine parathyroid hormone were purchased from Beckman (Palo Alto, CA, U.S.A.).

Preparation of isolated glomeruli [8]. Rats weighing approximately 120 g (Charles River strain) were anesthetized with ether. Bilateral nephrectomy was performed without renal artery perfusion. The outer cortex was dissected, immediately cooled to 0°C, minced with a razor blade to paste-like consistency, suspended in 1/15M phosphate buffer, pH 7.5, and rendered isoosmolar to plasma by addition of NaCl (0.05M) at 0°C. The suspension was centrifuged at 1200 × g for 90 sec, the supernatant discarded and the pellet resuspended after passage through a 25-gauge hypodermic needle (Sherwood Medical Industries, Ref. G 25 × 15/16). This procedure was repeated five times. The final pellet contained isolated glomeruli without Bowman's capsule (Fig. 1). The preparation of isolated glomeruli was used immediately for either binding or glomerular vasoreactivity studies.

Binding experiments. The binding of ^{125}I-angiotensin II to isolated rat glomeruli was studied at 22°C using 60 μg of glomerular protein in each tube. The incubation medium consisted of 200 μl of 1/15 M phosphate buffer, pH 7.5, in 0.05 M NaCl with 2% bovine serum albumin and 125 μg/ml of 1–24 adrenocorticotropic hormone (ACTH) fragment (Ciba Geigy, Basel, Switzerland). This latter peptide was added to inhibit the inactivation system of angiotensin II without affecting binding kinetics [9]. Standard incubation time was 12 min. Glomerular bound and unbound radioactivity were separated by filtration through a filter (Millipore, HAWP 02500). ^{125}I was determined with a crystal-type scintillation detector of 30% efficiency and specific binding calculated by subtracting binding in the presence of a high concentration of unlabelled angiotensin II (10^{-6} M) from total binding. Protein determinations were performed using the method of Lowry et al [10]. Data given in Figs. 2 to 5 represent the mean of four determinations. The SD was less than 10% of the mean in all cases.

Degradation of bound hormone was assessed by studying binding to fresh membranes and by polyacrylamide gel electrophoresis. Bound hormone was eluted in the presence of 0.05 M acetic acid (pH 3.2) and the eluate was neutralized with 0.1 M sodium hydroxide. This eluted radioactivity was then compared with intact ^{125}I-angiotensin II. From polyacrylamide gel electrophoresis, according to Corvol et al [6], the ratio radioactivity of the peak corresponding to ^{125}I-angiotensin II Rf over total radioactivity was calculated. Binding to fresh membranes at equilibrium was also determined for identical radioactive concentrations of intact tracer and eluted hormone.

Glomerular vasoreactivity experiments. Diameters of isolated glomeruli were measured with a micrometer under light microscopy at 22°C as a function of ^{125}I-angiotensin II concentration. At each hormonal concentration used, a test system of glomeruli suspended

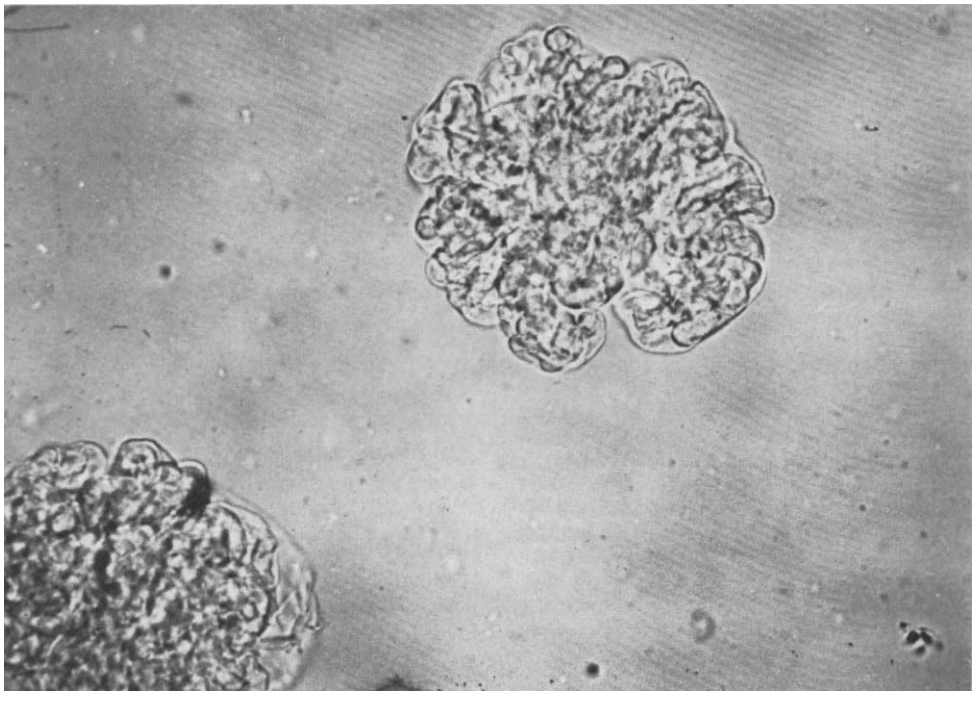

Fig. 1. *Photomicrograph of isolated rat glomeruli.* Only capillary tufts are present. Bowman's capsule has been eliminated (magnification, × 250).

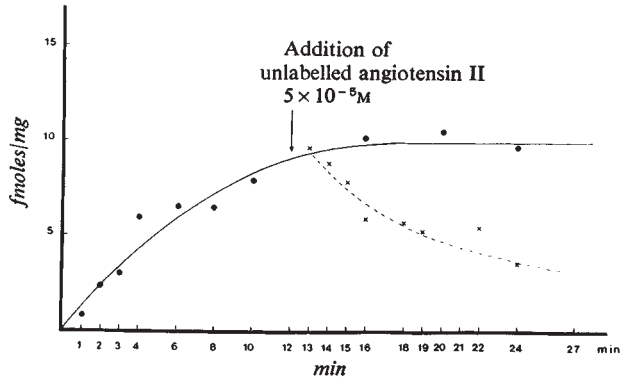

Fig. 2. *Uptake of ^{125}I-angiotensin II (1.7×10^{-10} M) by isolated rat glomeruli (closed circles).* After 12 min of incubation, unlabelled angiotensin II (5×10^{-5} M) was added in a parallel experiment (*crosses*).

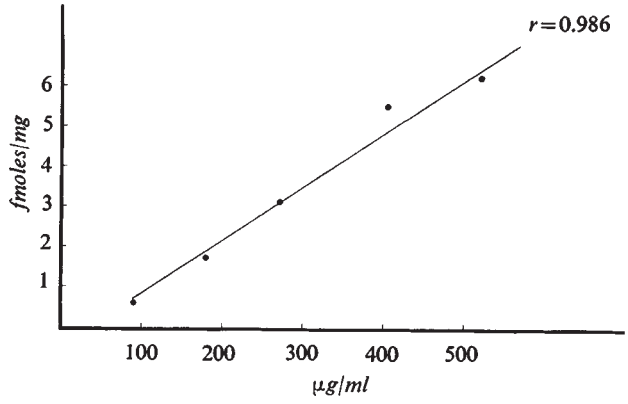

Fig. 3. *Binding of ^{125}I-angiotensin II (10^{-10} M) as a function of the concentration of glomerular protein.* The coefficient of correlation (*r*) of the regression line is shown.

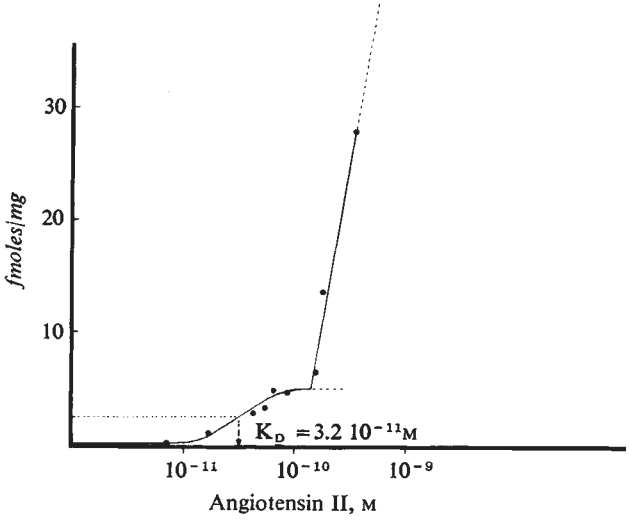

Fig. 4. *Binding of ^{125}I-angiotensin II at equilibrium (12 min) plotted against $\log_{10}$ concentration of monoiodinated purified ^{125}I-angiotensin II.* K_D for high affinity receptor sites is calculated as the concentration corresponding to 50% of the extrapolated plateau.

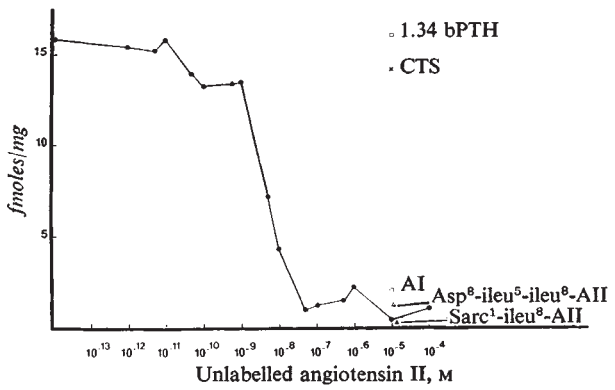

Fig. 5. *Inhibition of binding of ^{125}I-angiotensin II (10^{-10} M) in the presence of increasing doses of unlabelled angiotensin II.* The inhibitory effects of angiotensin I, asp⁸-ileu⁸-angiotensin II, sarc¹-ileu⁸-angiotensin II, salmon calcitonin (CTS) and 1–34 bovine parathyroid hormone (bPTH) at 10^{-5} M were also tested.

in 1/15 M phosphate buffer, pH 7.5, 0.05 M NaCl plus ^{125}I-angiotensin II was prepared and compared with a control preparation in the absence of hormone. Both preparations were examined after 12 min of incubation. The diameter of each of ten glomeruli was measured over approximately 1 min (11 min, 30 sec, to 12 min, 30 sec, incubation). The diameter of any given glomerulus varied according to the direction selected, but this was randomly chosen. Measurement of glomerular diameter at a specific given time (12 min) limited the number of measurements possible. Measurement of the diameters of ten glomeruli once only was preferred to several measurements of the diameter of fewer glomeruli. In order to avoid statistical bias, personnel preparing the solutions differed from those measuring glomerular diameters. Diameters of isolated glomeruli at 10^{-10} M were also measured under identical conditions as a function of time. Curves obtained using ^{125}I-angiotensin II were compared to those observed in the presence of unlabelled angiotensin II at identical concentrations. Percentage decrease of glomerular diameter related to glomerular diameter at zero dose or zero time was used as an index of glomerular vasoconstriction and, hence, glomerular vasoreactivity.

Results

Binding studies. ^{125}I-angiotensin II binding was measured as a function of time at varying hormonal concentrations between 5×10^{-11} M and 1.7×10^{-10} M. Equilibrium was reached after 10 to 15 min. Specific binding represented more than 95% of total binding. Nonspecific binding was entirely due to physical binding to the Millipore filters as measured by binding in the test system which did not contain receptor protein.

Glossmann, Baukal and Catt [9] in their studies on adrenal angiotensin II receptors have also shown that nonspecific binding was entirely due to physical absorption by the filter and that the presence of excess (10^{-6} M) hormone did not augment nonspecific binding. At steady state, addition of 5×10^{-5} M unlabelled angiotensin II produced rapid dissociation of the bound radioactivity from rat glomeruli (Fig. 2). The decrease in bound radioactivity represented release of ^{125}I-angiotensin II and not degradation of the tracer or receptor, or both, as evidenced by the persistence of the steady state in the absence of an excess of unlabelled molecules. Four time-course studies were performed. Association (k_1) and dissociation (k_{-1}) constants were derived once from the combination of two association curves and three times from the combination of an association and a dissociation curve. Assuming that the hormone binds reversibly to a homogeneous population of independent binding sites, the formation of the hormone-receptor complex (RH) is rendered by the following equation:

$$\log_e \frac{RH_{eq}}{RH_{eq}-RH} = (k_1H+k_{-1})t,$$

in which RH_{eq} is the concentration of RH at equilibrium and H the hormonal concentration in the medium. Since less than 5% of hormonal molecules are bound to the receptor at equilibrium, H can be taken as a constant. The dissociation curve can be assumed to follow the first-order rate equation:

$$\log_e \frac{RH}{RH_{eq}} = -k_{-1}t.$$

A good linear fit was obtained when these equations were applied to the experimental data. The correlation coefficient r was 0.987 ± 0.010 in five association studies and 0.944 ± 0.018 in three dissociation studies (mean ± SD). Calculations of k_1 and k_{-1} from the slope of the regression lines gave the values $k_1=0.254\pm0.078\times10^{10}$ M^{-1} min^{-1} and $k_{-1}=0.102\pm0.018$ min^{-1}. The ratio $k_{-1}/k_1(K_D)$ was $4.51\pm0.55\times10^{-11}$ M (mean ± SEM). The dissociation $t_{\frac{1}{2}}$ value ($\log_e2/k_{-1}$) was 6.8 min (Table 1).

^{125}I-angiotensin II binding at 12 min was linearly related to the amount of receptor protein in the assay medium at concentrations between 100 and 500 μg/ml (Fig. 3) and, thus, binding could be expressed as fmole/mg of glomerular protein.

After steady state was reached (12 min in the presence of 1.7×10^{-10} M ^{125}I-angiotensin II), bound hormone was eluted and examined by polyacrylamide gel electrophoresis and rebinding experiments. About 80% was undegraded when compared to the intact tracer (Table 2).

^{125}I-angiotensin II binding was measured as a function of hormonal concentration at equilibrium after 12 min of incubation (Fig. 4). An S-shaped curve was obtained for concentrations of ^{125}I-angiotensin II ranging from 10^{-12} M to 1.7×10^{-10} M, compatible with the presence of low capacity-high affinity receptor sites. At concentrations of ^{125}I-angiotensin II above 1.7×10^{-10} M, there was a rapid increase in bound radioactivity and this part of the curve probably reflected the presence of large capacity-low affinity receptor sites. K_D of the high affinity receptor sites could be calculated from the concentration of total hormone corresponding to 50% of the extrapolated plateau as free hormone always represented more than

Table 1. Indexes of binding

Experimental procedure	k_{+1}	k_{-1}	K_D[a]	K_D[b]	K_D[c]	*N* of binding sites
	M^{-1} min^{-1}	*min^{-1}*	*M*	*M*	*M*	*fmole/mg*
Hormone-receptor association kinetics for two hormonal concentrations	0.218×10^{10}	0.103	0.470×10^{-10}	—	—	—
Hormone-receptor association and dissociation kinetics at a similar hormonal concentration	0.474×10^{10} 0.213×10^{10} 0.109×10^{10}	0.149 0.093 0.064	0.314×10^{-10} 0.438×10^{-10} 0.583×10^{-10}	—	—	—
Hormone-receptor binding as a function of hormonal concentration	—	—	—	3.2×10^{-11} 15×10^{-11} 7×10^{-11}	1.24×10^{-10} 6.49×10^{-10} 1.41×10^{-10}	35.5 66.6 9.1
Mean ± SD	$0.254\pm0.156\times10^{10}$	0.102 ± 0.036	$0.451\pm0.110\times10^{-10}$	$8.4\pm6\times10^{-11}$	$3.05\pm2.98\times10^{-10}$	37.1 ± 28.8

[a] K_D derived from kinetic studies $\left(K_D=\frac{k_{-1}}{k_{+1}}\right)$.

[b] K_D derived from the concentration at 50% maximum binding

[c] K_D derived from Scatchard's plot (reciprocal of the slope).

Table 2. Degradation of eluted hormone after 12 min of incubation

	Binding to fresh membranes after 12 min of incubation at 5×10^{-12} M *fmole/mg*		Percentage of radioactivity recovered in the ^{125}I-A II[a] peak on polyacrylamide gel electrophoresis %
Intact ^{125}I-A II (a)	0.458	0.327	94.0
Eluted ^{125}I-A II (b)	0.352	0.280	79.3
Undegraded hormone, % $\left(\frac{b}{a} \times 100\right)$	76.9	85.6	84.4

[a] ^{125}I-A II, ^{125}I-angiotensin II.

95% of total hormone. This K_D was $8.4 \pm 6 \times 10^{-11}$ M (mean ± SD), of the same order as that calculated from time course studies. Scatchard's plot transformation was used to calculate the number of sites. The very low B/F (bound over free ^{125}I-angiotensin II) values made this calculation less precise than that derived from time course studies. K_D and number of sites were $3.05 \pm 2.98 \times 10^{-10}$ M and 37.1 ± 28.8 fmole/mg, respectively (Table 1).

Dilution of ^{125}I-angiotensin II (10^{-10} M) with increasing concentrations of unlabelled angiotensin II from 10^{-13} to 10^{-4} M decreased the percentage of labelled glomerular-bound hormone. Residual binding above 10^{-6} M was less than 10%. Angiotensin I, asparaginyl^1-ileu^5-ileu^8-angiotensin II and sarcosine^1-ileu^8-angiotensin II were all equally effective as competitive inhibitors of ^{125}I-angiotensin II binding. These two latter peptides are antagonists of angiotensin II with little or no intrinsic activity. On the contrary, salmon calcitonin and the 1–34 fragment of bovine parathyroid hormone did not inhibit binding when added at this same concentration of 10^{-5} M (Fig. 5).

Glomerular vasoreactivity studies. The percentage decrease of glomerular diameter after 12 min of incubation was plotted against $\log_{10}$ concentration of ^{125}I-angiotensin II. A sigmoidal effect vs. log dose curve was obtained (Fig. 6*a*). The calculated K_D (concentration of angiotensin II corresponding to 50% of the plateau) was 5×10^{-11} and 6×10^{-11} M in two separate experiments, respectively. These values are similar to those obtained when binding was plotted as a function of the log hormonal concentration (Table 2). A curve of the same shape was derived using unlabelled angiotensin II. The same K_D could be calculated (5×10^{-11}M)

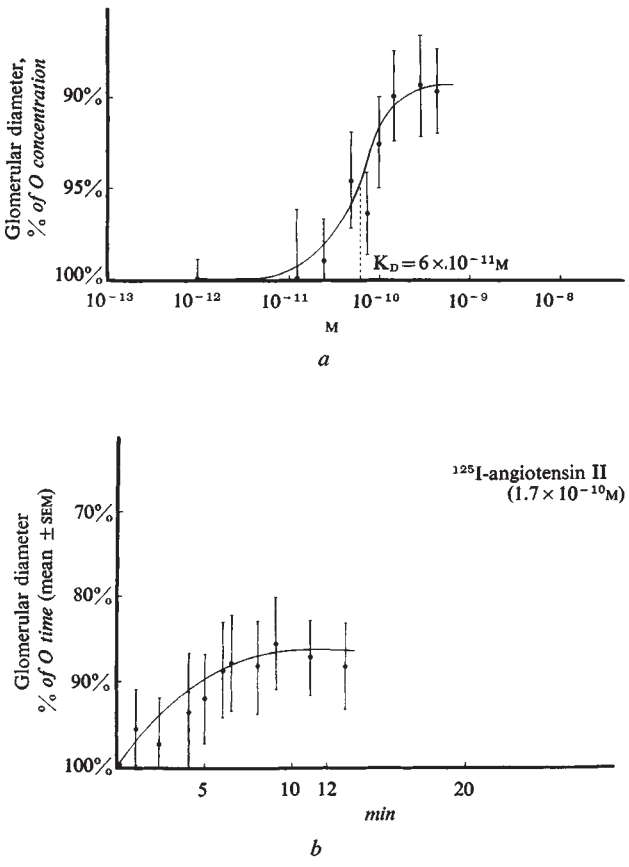

Fig. 6. *a, Percentage decrease of glomerular diameter at 12 min of incubation plotted against $\log_{10}$ concentration of monoiodinated purified ^{125}I-angiotensin II.* Each *point* represents the mean of ten determinations, and each *vertical bar*, twice the SEM. The percentage decrease is related to the glomerular diameter at zero concentration of ^{125}I-angiotensin II. K_D was calculated as the concentration of total hormone corresponding to 50% of the plateau. *b, Percentage decrease of glomerular diameter plotted against time in the presence of 10^{-10} M ^{125}I-angiotensin II.* Each *point* represents the mean of ten determinations, and each *vertical bar*, twice the SEM. The percentage decrease is related to the glomerular diameter at zero time.

demonstrating that labelled and unlabelled molecules display the same affinity for rat glomeruli. However, maximal decrease in glomerular diameter was slightly higher with unlabelled angiotensin II, indicating that the tracer had lost a portion of its biological potency. Time course studies plotting percentage decrease of glomerular diameter against time for a given ^{125}I-angiotensin II concentration were also performed. A plateau was reached between 10 and 15 min as in the binding studies (Fig. 6*b*).

Discussion

The specificity of binding of ^{125}I-angiotensin II to isolated rat glomeruli is demonstrated by the following:

very low calculated K_D, about 5×10^{-11} M, indicating high affinity of the hormone for its receptor; specific inhibition of binding of labelled angiotensin II by unlabelled hormone and by antagonists; reversibility of binding, independent of the degradation of either hormone or receptor; and only minor degradation of the eluted hormone which bound anew to fresh membranes demonstrating nonidentity of the receptor and catabolic sites.

The inhibition of binding by angiotensin I can be interpreted as due to transformation of angiotensin I to angiotensin II in the presence of converting enzyme which has been found in isolated glomeruli preparations [10, 11]. The precise localization of angiotensin II binding sites in rat glomeruli was not determined in the present study. Osborne et al [4] have suggested that they are located in mesangial cells.

A specific relation between binding sites as demonstrated in rat glomeruli and glomerular vasoreactivity is suggested by the calculated K_D (about 5×10^{-11} M, slightly higher than the physiological arterial plasma concentration in man [12] and probably in the range of values predicted in the rat from measurements of plasma renin activity [13]) and the close correlation between log-dose effect curve and log-dose binding curve from which similar K_D values were derived.

High affinity binding sites were amenable to study in these experiments because of the high specific activity of the tracer, 40 times that of tritiated angiotensin II. This probably explains the much lower K_D observed in the present study compared with that of Meyer et al [15], who demonstrated specific angiotensin II receptors in smooth muscle cells from the rabbit aorta. Conversely, our results are in the range of those obtained by Glossmann, Baukal and Catt [9] with ^{125}I-angiotensin II on high affinity receptors in the rat adrenal cortex, 1.85×10^{-10} M and 12 fmole/mg for K_D and number of sites, respectively. In order to compare the K_D found in the present study with the equilibrium association constant (K) observed by Glossmann et al, the reciprocal of K must be calculated as K is defined as the ratio k_1/k_{-1}. The ability of purified monoiodinated ^{125}I-angiotensin II to bind to receptors and produce physiological changes indicates that its effective biological potency is preserved and that the observed minor loss of potency is not of practical significance.

Acknowledgments

Professor F. M. Bumpus (Cleveland, Ohio, U.S.A.) supplied the ileu^8-angiotensin II used in this study; Professor D. Denton (Melbourne, Australia), the specific anti-angiotensin II antibodies; Ciba Geigy Cy (Basel, Switzerland), the α-L-asparaginyl^1-valyl^5-angiotensin II; and Armour (Kankakee, Michigan, U.S.A.), the salmon calcitonin. Drs. P. Corvol and J. Drouet performed the polyacrylamide gel electrophoreses, and Professor P. Meyer (Paris, France) provided help and advice. Dr. Judith A. Whitworth reviewed the English text. C. Lepreux provided technical, and M. Bizien, secretarial assistance.

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